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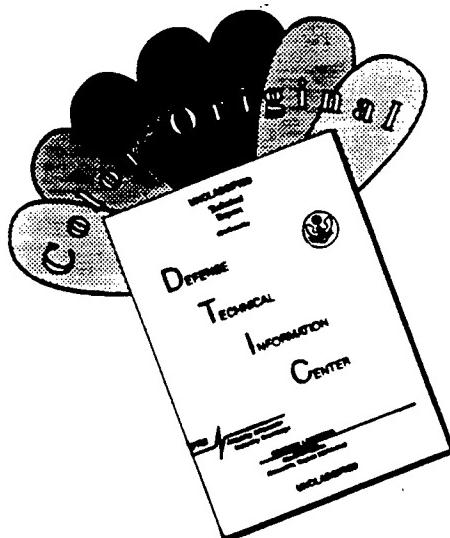
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During Phase I, IA, Inc. successfully demonstrated the feasibility of developing a strip test capable of distinguishing between levels of estrone-3-glucuronide·(E1-g) typical of a woman's fertile period and those typical of the non-fertile period. This level of discrimination will permit 72 hour advance notice of impending ovulation, making the strips a useful tool in family planning in that they provide sufficient advance notice of ovulation to allow for the lifetime of sperm in the vagina.

To implement this novel immunoassay technology, a new compound was synthesized and its ability to mediate an immunoassay was characterized using a fiber optic sensor. This immunoassay technology can be used to implement semiquantitative hormone assays by providing a distinct color change at specific points along a paper strip somewhat in the manner of a thermometer. The reagents are by nature more stable than enzymes and more amenable to rapid semi-quantification than colloidal gold. The test requires roughly 10 minutes to perform and was demonstrated to work in both buffer and urine samples. The new technology has potential for application to a large range of hormones, toxins, drugs and other analytes of physiological interest.

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Table of Contents

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4

Figures

Introduction

Project Summary

During Phase I, IA, Inc. demonstrated the feasibility of developing immunological test strips which provide a semiquantitative color indication of hormone levels in biological fluids at concentrations of physiological interest. The strips utilized an ionophore-linked antigen within a lipophilic membrane to transform immunological binding events into an alteration in concentration of ions transported into the membrane. Ion concentration changes were registered within the membrane as a color change produced by a chromionophore. The position on the strip where the color change occurred was used as a semiquantitative measurement of the concentration of the hormone, estrone-3-glucuronide (E1-g), which can be used to identify the period in which a woman is fertile. Test strips were able to distinguish a sample containing 20ng/ml of E1-g from one containing 80ng/ml. The height of a pink color rising up the strip immersed in the 80 ng/ml sample was clearly higher than that of the strip immersed in the 20 ng/ml sample as both strips were simultaneously exposed to their respective samples.

The Phase I project demonstrated the feasibility of the proposed technology. The two essential elements of feasibility were fulfilled: First, a molecule was synthesized which possessed both ability to act as an ionophore and ability to act as an antigen for an antibody to E1-g. These characteristics were observed quantitatively using a fiber optic sensor coated with the immunophore membrane used on the test strips. Second, test strips were developed for E1-g, the more difficult of the two hormones originally proposed. When used with urine samples, these test strips displayed visible color changes which identified the sample as containing either E1-g levels typical of fertile or non-fertile periods. Equally sensitive test strips for the second hormone, pregnanediol glucuronide (Pd-g), should be easier to develop because it exists in urine samples at concentrations roughly 100 time more concentrated than E1-g.

Demonstration of Feasibility of the Technology

Immunophore Test Strips were able to distinguish ovulatory from nonovulatory levels of E1-g in both buffer and urine sample roughly 75% of the time. Instances of inaccurate results were associated with visible differences in the way samples chromatographed up the strip. Control of chromatographic factors will be one of the focuses of Phase II work. In the example shown in Figure 2, the pink color in the ovulatory (80 ng/mL) sample was clearly higher than that of the strip simultaneously immersed in the non-ovulatory (20 ng/mL) sample.
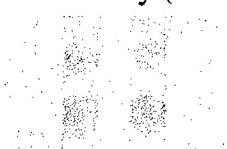
20 80

Figure 1: Phase I E1-g test strips could distinguish between non-ovulatory (20 ng/mL) and ovulatory (80 ng/mL) urine and buffer samples.

Measurements using fiber optic sensors coated with the immunophore membrane validated both the ionophoric and antigenic nature of the synthesized immunophore. These measurements lend weight to the claim that the color changes observed result from the measurement of E1-g by the immunophore membrane.

Test strips for the second hormone, pregnanediol glucuronide (Pd-g), which exists at concentrations roughly 100 time more concentrated than E1-g, were not constructed during Phase I but should be within the scope of the demonstrated technology because urine samples can be diluted if necessary. Given that time and resources proved insufficient to create strips for both hormones, success with the more physiologically dilute of the two hormones demonstrated the feasibility of the technology for the desired application.

The Nature of the Immunophore Test Strip Technology

The test strips developed during Phase I are based on a new immunoassay technology, the Immunophore Test Strip whose operation is illustrated diagramatically in Figure 1. The development of an Immunophore Test Strip requires synthesis of an immunophore molecule having one portion which transports a specific ion (e.g. K^+) and one portion which binds an antibody for the analyte to be measured. The Immunophore Test Strip consists of a semi-liquid membrane matrix (e.g. PVC/DOS) containing a mixture of chromionophores, lipophilic anions and immunophores. The chromionophores are lipophilic molecules which are blue when protonated and pink when unprotonated. Lipophilic anions maintain charge balance within the membrane. Immunophores on the membrane surface capture specific ions from the solution outside the membrane and transport them into the interior of the membrane. Because charge balance must be maintained and the number of lipophilic anions is limited, this causes deprotonation of some of the chromionophores, resulting in a shift in membrane color toward pink. However, when a surface immunophore comes into contact with the antibody it was designed for, it becomes reversibly bound to the antibody.

Immunophore Membrane

Chromatographic Strip

Symbol		Symbol	
$\oplus$	Protonated Chromionophore	$\odot$	Immunophore Transporting K^+
$\oslash$	Deprotonated Chromionophore	■	Antibody
$\bigcirc$	Immunophore	■$\bigcirc$	Antibody Bound to Immunophore
●	Potassium Ion (K^+)	∇	Antigen
		↓	Antigen Bound to Antibody
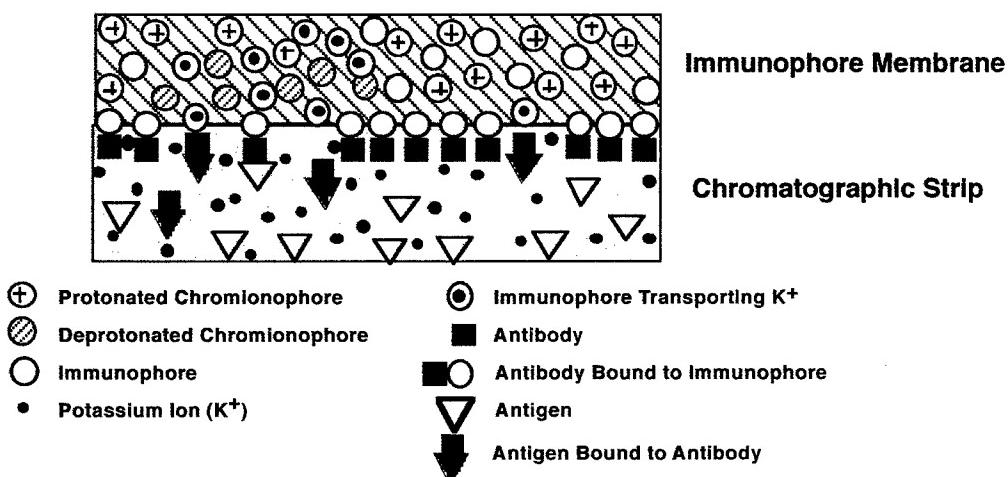
Figure 2: The membrane turns pink when urinary E1-g removes antibodies bound to surface immunophores which in turn transports K^+ ions into the membrane.

As long as the immunophore remains bound to the antibody, it remains immobilized on the membrane surface and can not transport ions from the solution into the membrane's interior. This shifts the membrane color back toward blue because more chromionophores are protonated to maintain charge balance in the membrane.

To prepare an immunophore strip for performing a semi-quantitative assay, the membranes is placed in contact with a limited amount of the antibody to the analyte which will be measured. A pH buffered mixture of the analyte to be measured and a known amount of the ionic species to be transported into the membrane is then placed on the wicking strip and transported to the membrane. As the sample chromatographs up the strip, the analyte competes for antibody bound to the surface immunophore. When sufficient competition has occurred the antibody bound to immunophore will be removed and the strip will turn from blue to pink because the immunophore will transport ions into the membrane. When samples containing different amounts of analyte are chromatographed over a membrane for fixed distance, the height to which the pink color extends is proportional to the amount of competing analyte in the solution.

This effect can be amplified by treating individual segments of an immunophore strip with increasing concentrations of antibody as one moves up the strip so that an assay strip can be developed in which color changes at specific locations on the strip will indicate a larger range of different concentrations of the analyte present in the wicking strip.

The Need for a Semiquantitative Test Strip for Hormones

Although test strips exist for many urinary metabolites, those metabolites which are found at concentrations below $10^{-5}M$ are not amenable to available direct color reaction methods. Concentrations of most circulating hormones and their metabolites are generally lower than this, consequently laboratory measurements based upon immunological binding are usually necessary. In the case of some hormones, such as human chorionic gonadotropin (HCG), and luteinizing hormone (LH), the clinically significant difference in hormone level is great enough so that color tests utilizing colloidal gold or enzyme mediated color changes can be used to produce over-the-counter (OTC) tests. These tests are not quantitative or even semiquantitative, but simply provide a yes or no test. In most cases, the question of interest is more subtle, requiring at least semiquantitative determination of the hormone concentration.

An illustration of this is seen in the case of the circulating steroids which regulate women's fertility cycles. The fertile period has been defined as the time during each menstrual cycle when intercourse may lead to pregnancy. It has been deduced from retrospective study of family planning records that greater than 90% of conceptions occur within a five day period which spans from three days before the peak of luteinizing hormone (LH) until two days after the peak.[1] The maximum notification of impending ovulation provided by measurement of the LH surge is 36 hours, while the average longevity of sperm in the vagina is 48 hours.[2] This means that tests predictive of ovulation which are based upon detection of the LH surge are useful for facilitating conception, but not for preventing it. Currently available OTC ovulation tests including the *Organon-LH-Color Test, Ovustick, Ovuquick, Test-pack,* and *Clearplan,* base their determination on detection of the LH surge and are therefore not appropriate for application to natural methods of birth control.[3-6]

The 17ß-estradiol metabolite, estrone-3-glucuronide (E1-g), reaches 85-95% of its peak value in urine 72 hours prior to ovulation.[2] It would, therefore, provide sufficient advance notice of ovulation to be a useful tool in natural contraception. The reason why an OTC test is not already on the market is that E1-g's more gradual monotonic peak requires a more quantitative detection device than the on or off color test currently employed in OTC LH tests. A multinational study by the World Health Organization Task Force on Methods for the Determination of the Fertile Period was conducted on women in 10 countries to determine the usefulness of E1-g assays as an aid to natural methods of birth control. The Task Force, which employed laboratory assays for E1-g, concluded that if the determination of E1-g could be made accessible to the home user, it would find great usefulness in the practice of family planning by periodic abstinence.[2] The possibility of error based upon this method arises on occasions when a premature E1-g rise meets a not fully ripened follicle. In that case the follicle will undergo atresia and a new one will be ripened which might produce ovulation following a second E1-g rise. Error due to this event can be avoided by also determining the rise of pregnanediol glucuronide (Pd-g) before declaring a safe period. Pd-g does not rise if ovulation has not yet occurred.[1, 7] The two hormones taken together should describe the boundaries of the fertile period.[1, 2, 7] The developmental effort which is herein reported will lead to an improved ovulation prediction system based upon semiquantitative measurement of E1-g and Pd-g in urine. It will also provide a technology which may be applied to other hormones and metabolites as well as drugs and toxins.

The development of a strip test technology which provides immediate semi-quantitative information about molecules which currently rely on laborious immunoassay methods will find many applications in hospitals, physicians' offices, at home and in the field. Currently, samples must be sent to laboratories where the use of expensive equipment and employment of laboratory technicians makes such determinations relatively expensive. The necessity for sending a sample to another location for assay also creates an inconvenient time delay in the availability of results. Many tests are simply unavailable under conditions frequently encountered in the field. A simple, quantitative, inexpensive office, field, or home monitor will overcome both of these difficulties. Cost of care will be reduced by elimination of expensive laboratory methods and care will be improved by virtue of real time availability of diagnostic information. This will be particularly valuable in third world settings where laboratory facilities

for such measurements are unavailable.

The Technical Basis for the Development

The innovation in this project is the combining of the mechanisms of ion selective optodes described below with those of immunoassay to yield a visual color response which is useful in a semiquantitative strip test. Other possible methods of producing a color response include the use of colloidal gold or enzyme-linked antibodies. Using colloidal gold, simple detection of antigens which would not be present except under the condition being tested has been reported with sensitivity at the nanogram to picogram level.[8-10] In these tests a positive response is seen as a pink spot. These tests were not semiquantitative, but were yes-or-no detection tests. Colloidal gold has been used in a quantitative way using instrumentation such as a densitometer.[11] However, for visual quantification, it takes some training to distinguish between shades of pink. For this reason it is not the most ideal method for a visual semi-quantitative strip test.

The strategy of linking an enzyme such as horseradish peroxidase or alkaline phosphatase to an antibody and observing the color change in a substrate is well known. Like colloidal gold, it's applicability to a semi-quantitative strip test is limited by the difficulty in comparing differing intensities of the same color. In addition, the reaction must be quenched to stop enzyme activity before reading, adding complication to the procedure. Additional drawbacks for field applications are that enzymes are not as stable as might be desired (short test kit shelf life) and . both colloidal gold and enzyme assay procedures require intervening rinse steps.

The semi-quantitative strip test which is being developed under this proposal uses a distinct transition from one color to another, such as that commonly seen with pH indicators, as the visual signal which must be recognized. Quantification of the response was accomplished spatially in two different manners, one similar to that of a thermometer using a continuos membrane field; the other using a series of spots of membrane which can be counted. The person reading the strip compares the location along the strip where the color change occurs with a calibration scale on the side of the strip. The method requires no rinse steps and the components of the strip are considerably more stable than enzymes.

Ionophore Membrane Technology

Recent research has turned to the development of natural and synthetic ionophores as ion selective components in electrodes for potentiometric determinations of various ions. Such molecules can transport a specific ion through a lipophilic membrane, leaving other ionic species behind. The theoretical basis for ion selectivity has been described by Erne et al.[12] These ionophores are lipophilic molecules which selectively chelate the ion by virtue of the geometry of placement of sulfur, oxygen and/or nitrogen atoms in a C-shaped molecular structure of appropriate size.[13, 14] Design of such molecules having sufficient lipophilicity so as to not wash out of the membrane (log P_{TLC}=17.5) has been described in the literature.[15]

These ionophores cannot carry cations through a single component ionophore membrane by diffusion. An electrical potential or pH gradient is required to pull the positively charged ion to one side of the membrane while leaving its negative counter-ion behind. Although this can be accomplished easily in electrode-based systems, it presents an obstacle in optical detection systems. To overcome this problem, tetrakis (4-chlorophenyl)borate, a lipophilic anion which balances charge within the membrane, has been used to create a dual-component ionophore membrane which functions as an ion-exchanger. This eliminates the need for an electric potential to facilitate passage of the positive ion.[15] Improvement of this technique was demonstrated using potassium tetrakis[3,5-bis(trifluoromethyl)phenyl} borate.[16]

The final element needed to make application of ionophore membranes to visual measurements possible was the development of chromionophores which change their optical characteristic upon binding of a particular cation, and which are lipophilic enough to remain within a membrane.[17] Chromionophores have allowed ion selective optodes to be developed for various cations.[18-22] A Pb^{+2} sensitive ionophore based optode has been developed[23] which

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9

demonstrated an ability to measure lead concentrations in water between 5×10^{-9} M and 5×10^{-3}M with a precision of 10%, a far greater sensitivity than had been possible with the lead selective electrodes. This membrane functioned in the manner of an ion exchanger. The operation of this type of membrane is as follows: In the absence of Pb^{+2} the anions are available to the chromionophores so that H^+ is transported into the membrane and the colored chromionophore absorbs light radiation. When lead is present, each Pb^{+2} ionophore associates with two anions (charge neutrality condition) making them unavailable to accompany the chromionophores. The number of colored chromionophores in the membrane is reduced and less radiation is absorbed.

What allows the optode to achieve sensitivity at lower concentrations than can be achieved with similarly based ion-selective electrodes is that the color change is mediated by a lipophilized pH indicator. For the sake of simplicity of explanation let us assume that such a pH indicator is responding to H^+ concentration within the membrane resulting from competition with an ion being transported by a similar concentration of an ionophore having a K_a for its ion which is similar to that which the chromionophore displays for H^+ ion. By choosing a pH indicator which changes around pH 9, the critical concentration where color change occurs results when the other ion is transported in a concentration $\geq 10^{-9}$M. By choosing a pH indicator which changes around pH 6, the ion concentration where color change occurs is $\geq 10^{-6}$M. Such a system gives great flexibility of design in that the color change can be made to occur at the concentration of interest for a given analyte. In actual practice the response curve of a cation selective optode is described by the equation given below,[24] and the K_a and concentrations of the chromionophore , anion and ionophore are not identical. However, the principle is easier to grasp with the aforementioned simplifying assumptions.

$$a_{iv} = (K_{exch}^i)^{-1} \left(\frac{\alpha a_{H^+}}{1-\alpha}\right) v \frac{[R_{tot}^-]-(1-\alpha)[C_{tot}]}{v([L_{tot}]-\frac{P}{v}\{[R_{tot}^{-1}]-(1-\alpha)[C_{tot}]\})^P}$$

where: $K_{ex} = \frac{k_{K^+}}{k_{H^+}} \beta_{K^+L} K_a$

R_{tot}^{-1} = the concentration of anionic lipophilic sites within the membrane

C_{tot} = the concentration of chromionophores is the membrane

L_{tot} = the concentration of ion-selective ionophore in the membrane

v= the charge on the selected ion

a= equilibrium activity of ions, and

$\alpha = \frac{[C]}{[C_{tot}]}$ P= number of ionophores in metal-ionophore complex (as MI_P)

$\frac{k_{K^+}}{k_{H^+}}$ = the distribution coefficients of the uncomplexed H^+ and K^+ ions between

the aqueous phase and the membrane phase

β_{K^+L}= the stability constant of the ionophore-potassium complex

K_a= the acidity constant of the chromionophore

IA, Inc. has been working on a project developing a fiber optic lead sensor based upon these principles. Dr. Eric Bakker, an author on the above referenced paper, has been serving as a

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10

consultant in this project and has provided us with software which allows design of such systems based upon these equations. Dr. Bakker is also serving as a consultant on the project herein reported.

Linking Ion-Selective Membrane Response To Immunological Binding Events.

The best methods for measuring hormones all rely upon measurement of binding between a hormone-specific antibody and that hormone in a sample. Antibodies having $K_d=10^{-10}$ are not uncommon, making measurement of very low concentrations of hormone possible. The technology herein reported utilizes this principle, but in a new way. Instead of an immunoassay based upon radioisotopes (RIA) or enzyme linked antibodies (ELISA) this is a new type of visual immunoassay, one based upon "immunophores". An immunophore is herein defined as an ionophore which has been conjugated to an antigen. By virtue of this conjugation, the availability of the ionophore for participation in the ion-exchange equilibria with the chromionophore and the lipophilic anion within an ion selective membrane is altered by binding of the antigen to an antibody at the surface of the membrane. In terms of the equation previously presented,

$$K_{ex} = \frac{k_{K^+}}{k_{H^+}} \beta_{K^+L} K_a$$

the value of β_{K^+L} will vary as a function of antibody binding. Antibody binding will in turn vary as a function of the amount of competing antigen present in a sample.

While the idea of using an immunophore in the context of an ion exchange membrane containing a chromionophore is new, the idea of creating an immunophore is not. Such molecules have been created for the purpose of making immuno-electrodes which have been reported in the literature.[25-27] In this context, the immunophore allowed the electrode to be responsive over the range of 10-150 µg/ml of antibody or competition by 1-100nM antigen. In one case, the antigen was digoxin, which, like E1-g and Pd-g is a steroid.[25]

Although building upon the immunophore technology reported for electrodes, the test strip is quite a different type of measurement tool. It consists of an absorbent medium, such as filter paper, which has been coated on one side with a lipophilic membrane containing chromionophore, immunophore and lipophilic anion. Antibody solution is spotted onto the strip below the membrane and dried. Sample is mixed with a diluent containing antibody, a pH buffer and a high concentration of ion (so that the concentration of the ion being sensed is not altered by the ions in the sample). The strip has a color corresponding to that of the protonated chromionophore when L_{tot} is at its maximum. The bottom of the strip is placed in a specific volume of the diluted sample and the liquid allowed to rise chromatographically up the strip. As it rises, antibody which has not been bound by antigen in the sample will bind to the immunophore on the strip, causing the chromionophore to change color. At some point on the strip, a sufficient amount of antibody will have been bound so that the amount remaining which binds to that region of the strip is not sufficient to cause the chromionophore to change color. This point will divide the strip into two color regions. The amount of antigen which causes this to occur at various points along the strip will be calibrated so that the concentration can be read by the division point of the color of the strip.

The feasibility of developing semiquantitative test strips based upon this immunophore technology has been assessed through the objectives of Phase I of this proposal as applied to the hormone E1-g. The technology appears feasible and can potentially be applied to many other molecules including other hormones, neurotransmitters, drugs, toxins and critical metabolic markers for specific illnesses. The results of Phase I work are reported in the following section.

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11

Body of Report

Task I: **Construct A Strip Which Provides A Visible Response To Changes In Ion Concentration.**

> In this task, a paper strip coated with a membrane containing chrom-ionophore ETH 5294, lipophilic anion, and ionophore benzo-15-crown-5 changed color in response to addition of 0.5 M potassium ion. This validated the use of benzo-15-crown-5 as the ionophore part of the immunophore to be synthesized and established the initial amounts of membrane components to be used for making immunophore test strips.

Materials and Methods

Fluka Chemika is the primary supplier of components for ionophore membranes. Of the commercially available chromionophores, three of them (ETH 5294, ETH 2349 and ETH 5350) are derivatives of the pH indicator, Nile Blue A. The fourth chromionophore (ETH 2412) exhibits different optical characteristics. ETH 5294 and ETH 2412 were initially tested to determine the minimum concentration of chromionophore which could be used in the membrane.

A membrane solution was prepared using 20 mg of high molecular weight poly(vinyl chloride) (PVC)(*Fluka*), and 40 mg of bis(2-ethylhexyl)sebacate (DOS), in 1 mL. of freshly distilled tetrahydrofuran (THF). To this was added varying amounts of ETH 5294 and amounts of the lipophilic anion, sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate, and potassium ionophore, benzo-15-crown-5, sufficient to create a 1: 1.1: 10 ratio of chromionophore: anion: potassium ionophore. Response of the strips to varying conditions of pH and KCl concentration were examined. Several different grades of chromatography paper were tested as a matrix for application of the ionophore membrane and the time required for buffer to chromatograph up the strip was measured.

Other media tested for supporting the membrane including nylon mesh and C-18 reversed phase silica chromatography plate. Also tested were membranes of identical composition except that PVC was replace by an equal amount of polyurethane.

Results

Solution containing 2.9×10^{-6} mmole of ETH 5294 per mg of plastic (PVC/DOS) coated onto a piece of filter paper by simple dipping described the minimum concentration of that chromiono-phore which gave a easily visible change from blue to pink upon application of 0.5M HCl or NaOH respectively. Response to this application occurred within 30 seconds, turning the strip pink in response to base and blue in response to acid.

A control strip inserted into the pH buffers without KCl was pink at pH 8.5, but turned blue in pH 7.5 as well as pH 6.5. A second strip when placed in a similar solution containing 1M KCl, was pink at pH 8.5 and pH 7.5 and blue at pH 6.5. pH 7.00 and a KCl concentration of 0.5M were selected as giving a clear distinction between the pink color resulting from baseline ionophore concentration and the blue color produced when ionophore concentration is reduced (as will effectively occur when antibody binds the immunophore). This places K^+ concentration in sufficient excess over what is required so that the color change will not be altered by fluctuations in urine K^+ concentration.

The color change required 5-10 minutes when the sample was chromatographed up chromatography paper (grade 20) coated with a membrane of previously described composition. Other media were tried under similar conditions and were found to be less effective than the grade 20 chromatography paper. When a nylon membrane was used in place of the chromatography paper, the color change was not as easy to see. Use of a matrix of C-18 silica

on glass destroyed the responsiveness of the membrane to pH change.

Substitution of polyurethane in place of PVC resulted in a more hydrophilic strip which responded similarly to the PVC strip. Use of ETH 2412 in place of ETH 5294 was also examined, however the color change from green to pinkish-yellow was less striking so it was not pursued further.

Discussion:

These experiments determined that the conditions for making the initial tests of the immunophore to be synthesized will be 2.9×10^{-6} mmole of ETH 5294 per mg of plastic (PVC/DOS) with anion and immunophore in molar ratios with respect to ETH 5294 of 1.1 and 10, respectively. Tests will be in a solution of .5M KCl in 0.1M, pH7 tris buffer. When subsequent computer simulations were carried out in Task III, this information will be used to define the minimum value for chromionophore concentrations employed in the design calculations. When test strips are made chromatography paper (grade 20) will be used.

These experiments completely fulfilled the objectives proposed under Task I and established the feasibility of creating an ionophore membrane on a test strip which yields clearly visible changes to differing conditions of pH and ionic strength.

Task II: Demonstrate Transduction of a Color Change By Means of an Immunophore.

> This task was carried out twice. Although the first synthesized immunophore demonstrated the ability to transport potassium and bind to anti-E1-g antibody, it washed out of the test strip membranes. The second synthesized immunophore demonstrated potassium transport at pH 5.28 and antibody binding at pH 7. It transduced a color change in test strips. It did so, however, at a pH 5.28, which was incompatible with the antibody.

Experimental Methods and Results

Measure Immunophore Antigenicity with Fiber Optic Sensor

<u>Apparatus - Sensor Cartridge</u>
Each fiber biosensor is contained within a sensor cartridge as shown in Figure 1.
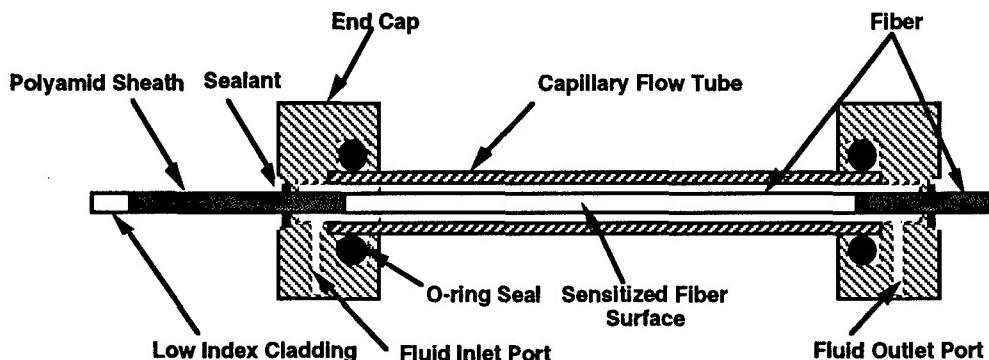
Figure 3:   A fiber optic sensor was used to measure the antigenicity of the immunophore.

400µm step indexed, multi-mode, fused silica fiber was cleaved using a York Electronic Fiber Cleaver to yield 10 cm pieces with optically acceptable end faces. Pieces were stripped of cladding and given a proprietary treatment to inhibit non-specific binding of proteins. Following this, the ends were coated with a low optical index material (n≈1.3) to constrain the

light at the points where the fibers contact the sensor flow cell. This coating is protected by placing a protective black polyimide sheath around the low index end cladding.

The bare fiber surface was chemically coupled to estrone-3-glucuronide as follows: Silanization of the fibers was performed in a batch fiber processing unit which was designed for the purpose of performing chemistry on a batch of fibers. This was performed in a glove box under nitrogen to prevent hydrolysis using a method similar to that reported by Bhatia, et al.[28] The longitudinal surface of the fibers was surrounded by a 2% solution of 3-(mercaptopropyl)-trimethoxysilane in dry toluene for 2 hr at room temperature, creating a glass surface bearing thiol groups. After rinsing in toluene, the thiol groups on the fibers were reacted with the maleimido moiety of the heterobifunctional agent, γ-maleimidobutyric acid-N-hydroxy-succinamide ester (GMBS) by incubating them in a 2mM solution of GMBS in reagent alcohol for 1 hr. The succinimide ester of the GMBS was then coupled to hexane diamine by incubating the fibers for 4 hours with a 1 mg/ml solution of hexane diamine in pH 9.3 0.1M carbonate buffer. After rinsing with water, the fibers are incubated overnight with a solution containing 5 mg/ml of E1-g and 10 mg/ml of 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) in water at pH 4.2. Fibers are then rinsed with water and stored dry.

Handling the fiber only by its clad ends, the sensitized fiber was inserted a capillary tube without touching its sensitized surface. End caps were then placed over the fiber ends, o-rings pressed in place, and finally sealant was placed around the points where the fiber passes through each end cap. Once assembled, the fiber rests securely within a glass micro capillary tube having a fluid volume of ≈80 µL. After manufacture, the sensor cartridges are stored at room temperature until needed. Then, a technician takes a sensor cartridge and connects it to fluid coupling tubes in the cartridge mounting block on the fluorometer.

<u>Apparatus - Fiber Optic Biosensor Fluorometer</u>
IA's fiber optic biosensor fluorometer was utilized for measuring sensor cartridge fluorescence. This fluorometer was constructed using post mounted Newport optical components and occupies a 2' x 4' optical bench. A simplified optical layout of the instrument is shown in Figure 2.
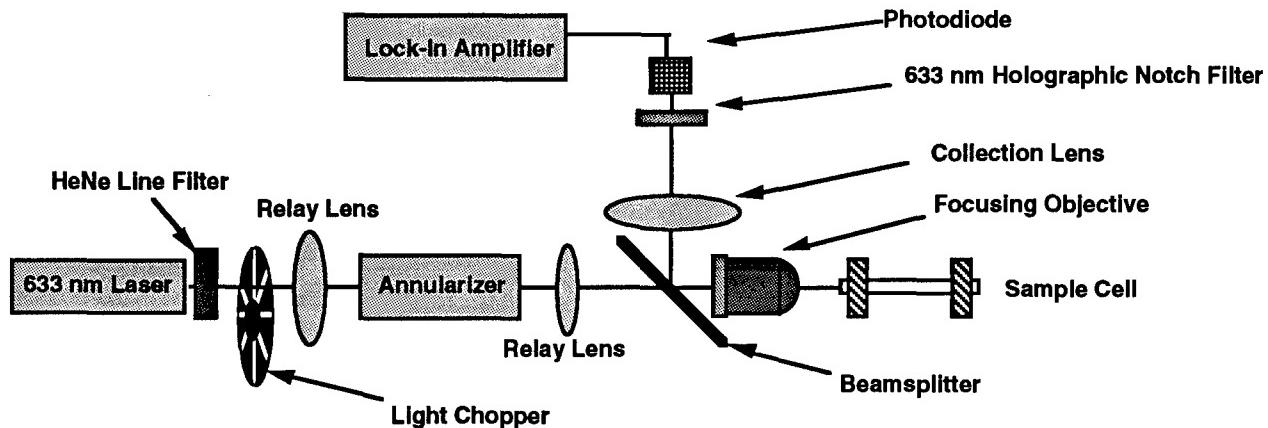
Figure 4: A benchtop breadboard fluorometer was used to measure membrane characteristics.

The fiber optic biosensor fluorometer uses 633 nm 7mW HeNe to stimulate fluorescence in the fiber sensors cartridges. To remove low-level radiation from the HeNe gas discharge, the HeNe beam is first passed through a 633 nm laser line filter. The beam is then modulated using an SRS Model 540 Optical Chopper. The synchronization output of the optical chopper is attached to the synchronization input on the SRS 810 lock-in amplifier. The beam axis and direction are adjusted using dogleg mirrors and the beam is passed through a mechanical shutter. This beam is then injected into an annularizing beam shaper. The output beam is collimated and passed through a 50/50 beam splitter into a high numerical aperture microscope objective (NA=0.55) which is used to inject the beam into the sensor cartridge.

The sensor cartridge is mounted on a V-block and is held in place using end cap clamps. Using the visible reflection from the front face of the fiber, the V-block position is adjusted using an XYZ micropositioner stage to place the sensor cartridge's input fiber face at the microscope objective's focal point and to center the fiber face around the focal point.

Broad band sensor cartridge fluorescence emitted at all angles from sensor's fiber surface propagates to the input face of the fiber where it is collected by the microscope objective and reflected by the 50/50 beam splitter into the collimating relay optics. The collimated fluorescence beam is then passed through a holographic notch filter which provides a factor of 10^6 rejection for any 6353 nm HeNe laser radiation which may have been scattered or reflected back into the optical signal path by the sensor cartridge or other optical surfaces. The fluorescence signal is finally focused onto a photodiode and measured using an SRS Model 810 Lock-in amplifier which is synchronized to the optical chopper.

IIa-1. Immunophore Synthesis #1

Background to decision to synthesize a different immunophore than was proposed:
The immunophore which was originally proposed to be synthesized for testing the feasibility of utilizing an immunophore to modulate the color response of a test strip was a conjugation between Pd-g and the potassium ionophore, benzo-15-crown-5. It was decided to begin work with estrone-3-glucuronide-15-benzo-crown-5 rather than with the originally proposed pregnanediol-3-glucuronide-15-benzo-crown-5 because of cost considerations. While the cost of both pregnanediol-glucuronide and estrone-3-glucuronide is $578/100 mg. Estrone-3-glucuronide can be synthesized in a fairly straightforward manner, giving us a substantial amount of starting material for synthesis of the subsequent immunophore, whereas it was less straightforward to synthesize pregnanediol-glucuronide.

Summary of synthetic method:
The first immunophore which was synthesized was an estrone-3-glucuronide-4-amino-benzo-15-crown-5 conjugated between the amino of the benzo crown and the carboxyl of the glucuronide. The first step was to synthesize estrone-3-glucuronide. To ensure that the correct position of the sugar reacted with the 3 position of the estrone, the starting materials were estrone and 1-bromo-2,3,4-acetoxy-glucuronic acid methyl ester. These were combined in dry toluene with dry cadmium carbonate in a nitrogen atmosphere. The reaction mixture was heated to distill off any water as a toluene azeotrope as the reaction proceeded for 30 minutes. The reaction mixture was filtered over a pad of celite, washed with toluene and concentrated under reduced pressure to yield the protected estrone-3-glucuronide. Protecting groups were hydrolyzed off with NaOH and the product purified by flash chromatography on silica gel. The purified estrone-3-glucuronide was combined with 1 equivalent of 4-amino-benzo-15-crown-5 and 1.2 equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride in water at pH 4.0. The mixture was stirred at room temperature for 48 hours and the resulting estrone-3-glucuronide-4-amino-benzo-15-crown-5 conjugate was purified by flash chromatography on silica gel. The immunophore was dried under reduced pressure and dissolved in methanol and tetrahydrofuran.

Testing Immunophore #1 for binding to anti-E1-g Antibody

> **As predicted, immunophore #1 binds to anti-E1-g.**

Materials and Methods:
Antibody recognition of the immunophore was compared to that of estrone-3-glucuronide (E1-g) using a fiber optic sensor which has been developed by IA for measurement of estrone-3-glucuronide. This sensor is routinely used in our laboratory and provides data on antibody binding much more quickly than the ELISA assay method originally proposed. The sensor apparatus and preparations of estrone-3-glucuronide sensitive fibers have been described on pages 5-7 of the Final Report on this project (Contract #DAMD17-96-C-6026) submitted under separate cover.

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15

Measurement Procedure: Anti-E1g monoclonal, clone #278-17144 was obtained from OEM Concepts. 1 mg of the antibody was labeled with the fluorophore Cy-5 (Amersham Life Science) according to the directions provided with the reactive fluorophore and purified by HPLC on Superex G-200. The labeled antibody was diluted with 0.1M phosphate buffer, pH 7.2, containing 8.7 g of NaCl, 1 g of BSA (Sigma 30% solution) and 0.5% Tween 20 so as to yield a concentration of 2.2×10^{-7}M antibody. This solution was in turn diluted 1:8 with the same buffer. 180 µl of the first dilution was mixed with 20µl of the sample. The more diluted antibody solution was injected into the sensor cell and the fluorescence gathering on the fiber was monitored automatically. Readings from this dilute antibody solution serves as a means of calibrating different fibers so that they can be compared. Following this, the calibration standard was removed and the sample-antibody mixture injected and fluorescence gathering on the fiber automatically recorded as before.

<u>Results</u>
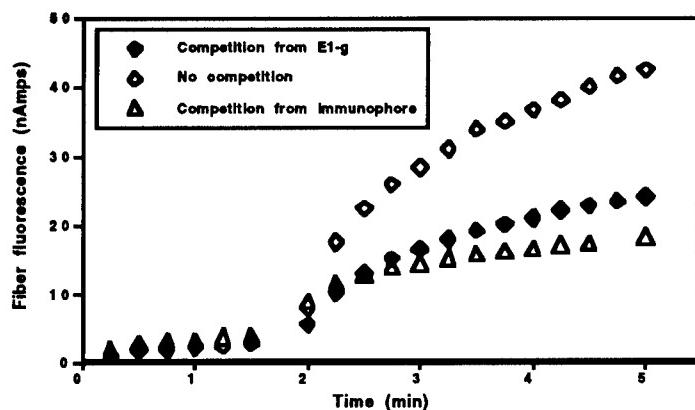
Figure 5: Immunophore # 1 inhibits binding of Cy-5-anti-E1-g to the sensitized fiber surface.

It is the nature of the sensor that without competition from free estrone-3-glucuronide or another antigen, the antibody bearing a Cy-5 fluorophore is attracted to an optical fiber bearing covalently attached estrone-3-glucuronide to yield an increasing fluorescent response by the sensor over time. The magnitude of this response is reduced by the presence of estrone-3-glucuronide in the sample. Figure 3 shows a comparison between the responses of fiber sensor having no estrone-3-glucuronide in the sample, one having 1.71 nM estrone-3-glucuronide and one having an equivalent molar amount of immunophore #1. The reduced response of fibers exposed to E1-g and to the immunophore indicates that the immunophore, like E1-g, acts as a competing antigen .

IIb-1. Fabricate Immunophore Test Strips

> **Although Immunophore #1 membranes changed color in response to potassium, Immunophore #1 washed out of the test strips. A more lipophilic immunophore was designed for subsequent synthesis.**

A membrane solution was made up as previously described except that Immunophore #1 was used in place of the ionophore. Membrane was coated onto strips as before. When KCl solution was chromatographed onto the strip, an area turned pink, as it had with the ionophore; however, in the case of the immunophore the pink area migrated up the strip along with the solvent front, leaving the strip blue behind the front. This indicated two things: that the immunophore retained the capacity of the potassium ionophore to change the color of the membrane in the presence of 0.5M KCl, and that it is so hydrophilic that it migrates with the aqueous phase rather than remaining in the membrane phase. While the former characteristic is necessary, the latter characteristic makes this ionophore unsuitable. It must stay in the membrane unless it is removed by binding to an antibody. It was therefore necessary to return to Task IIa and synthesize a more lipophilic immunophore.

IIa-2. Immunophore Synthesis #2

Immunophore #2, N-dodecyl-N-[estrone-3-glucuronide monoacetate]-4'-aminobenzo-15-crown-5 was synthesized. This synthesize was repeated twice to provide sufficient material for use in subsequent tasks.

Background to the synthesis and change in proposed workplan:
The approach to making a more lipophilic immunophore was to add a dodecyl chain to the amine through which the E1-g is conjugated to the 4-amino-benzo-15-crown-5. This had as a consequence that, because the coupling amine was now a secondary rather than a primary amine, the carbodiimide coupling method employed in synthesizing the first immunophore, was no longer effective. The coupling reagent was changed to BOP-Cl (Bis[2-oxo-3-oxazolidinyl]phosphinic chloride) because this reagent will couple a secondary amine to a carboxyl group. It brought with it, however, new difficulties in that it also acts as a coupling agent for hydroxyls, of which the glucuronide possesses several. It was therefore necessary to use an E1-g molecule in which the hydroxyls were protected. Commercially available acetobromo-α-D-glucuronic acid methyl ester, which was a fully protected glucuronic acid ester was therefore obtained and coupled to the steroid. It was then necessary to selectively deprotect the carboxy-methyl ester while maintaining protection of the hydroxyls. This required several attempts before the immunophore #2 was successfully synthesized. As a consequence, the original intention of also making an immunophore for creating a pregnanediol-glucuronide test strip could not be achieved during Phase I work, due to lack of time and resources. Development of the second test strip will be part of Phase II work. The successful synthesis of immunophore #2, N-dodecyl-N-[estrone-3-glucuronide monoacetate]-4'-aminobenzo-15-crown-5, is shown in Figure #4.

Synthetic Method: Estrone-3β-D-glucuronide triacetate **3** was prepared in 64% yield, as per published procedure[29] with slight modifications[30] by reacting estrone **2** with acetobromo-a-D-glucuronic acid methyl ester **1** [IR(KBr): 1758, 1498, 1226, 1100, 1053 cm^{-1}]. Methods attempted for selective deprotection of the carboxylic methyl ester included potassium carbonate / water in dioxan,[31, 32] potassium carbonate / water in methanol[33] and barium hydroxide octahydrate in methanol.[34] The later one afforded estrone glucuronic acid monoacetate, deduced from ^{1}H NMR and IR. [IR(KBr): 3421, 1727, 1498, 1245 cm^{-1}]. We are confident that by controlling the amount of the reagent, we can obtain selective deprotection of the methyl ester. The synthetic scheme is shown in Figure 4.

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17
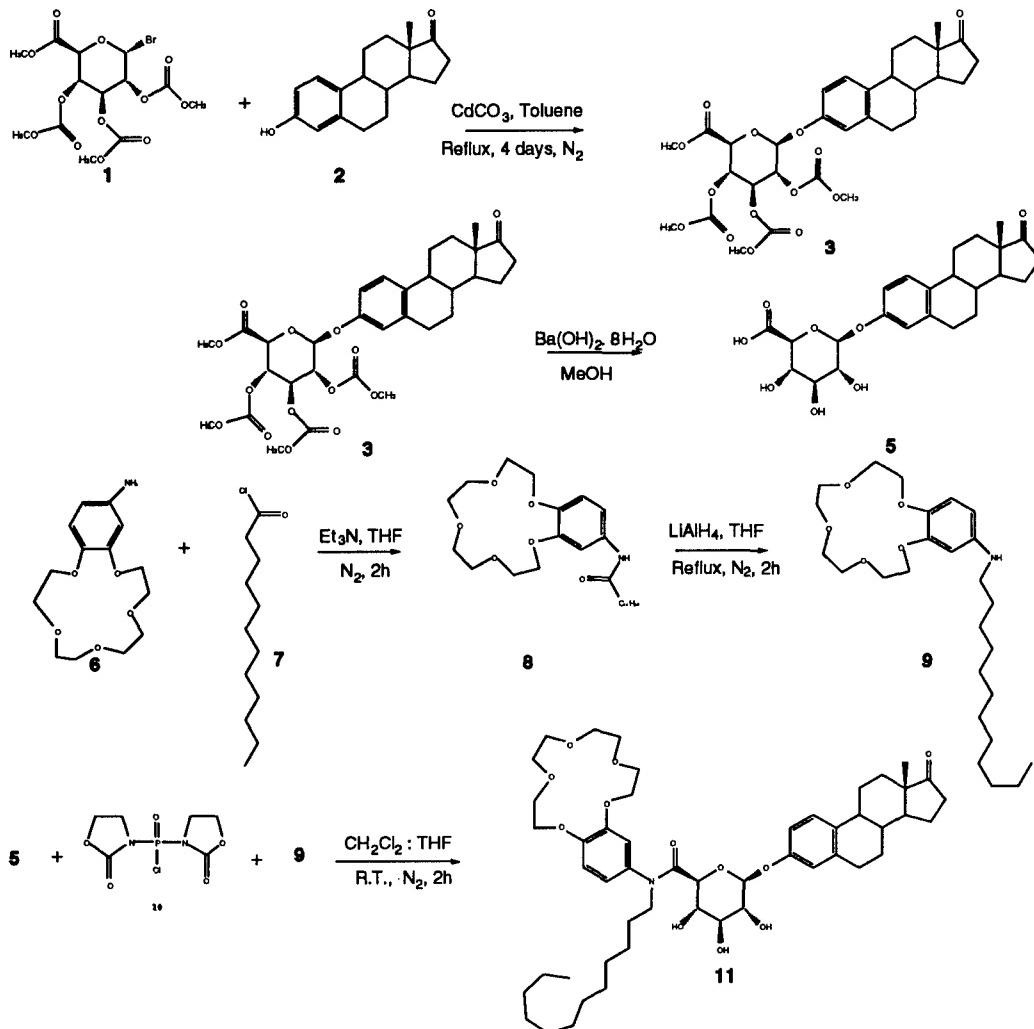
Figure 6: Synthesis of Immunophore #2.

The compound **9** was obtained[35] first by reacting dodecoyl chloride with 4-aminobenzo-15-crown-5 **8** in 91% yield [IR (KBr): 3448, 3286, 1656, 1141 cm^{-1}] and which on reduction with lithium aluminum hydride afforded **9** in ~85% yield [IR (KBr): 1618, 1130 cm^{-1}]. Compound **5** was coupled to **9** with BOP-Cl (Bis[2-oxo-3-oxazolidinyl]phosphinic chloride)[36] **10** in dichloromethane / tetrahydrofuran solvent mixture and in the presence of triethyl amine, to obtain **11**. The crude compound contains some unreacted starting dodecylamino benzo-15-crown-5 **9**, which was extracted in chloroform yielding a mixture of two compounds in ethyl acetate layers. This mixture was separated to obtain **11** and another unidentified compound. Compound **11** has characteristic IR stretching frequencies [IR(neat): 3385, 1736, 1653 cm^{-1}].

IIb-2: Testing Immunophore #2

> **While immunophore #2 acted as an ionophore for potassium ion, bound anti-E1-g, and transduced a color response on a test strip, it functioned at pH 5.28 rather than the desired pH 7.**

1. **Ionophore activity:** The new immunophore was incorporated into a membrane and applied to paper strips as described in Task #1. The membrane solution which was created as previously described using immunophore #2 in place of the ionophore. This solution created strips which behaved very differently. The pH 7 buffer which had turned previous strips blue in the absence of potassium, left the strip in a pink state. The strip did not turn blue until the pH dropped to

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18

5.28. Solutions of pH 5.28 magnesium acetate buffer (50 mM) were made to contain 4 levels of K^+: 0, 10mM, 100mM and 500mM. Administration of 0.3M KCl at this pH returned the strip to pink. The end of a membrane treated strips was placed in 100μl of one of the solutions and the color change was noted as the solution chromatographed up the strips. The strip without K^+ remained blue. The one containing 10 mM K^+ became purple. The ones containing 100mM and 500mM K^+ both turned pink. This established that the compound acts as an ionophore. The color did not migrate up the strip as had been observed with the previous immunophore. It is therefore a good candidate for use in the proposed technology.

<u>2. Binding to anti-E1-g Antibody:</u> Because of the success with which a lipophilic immunophore was created, the immunophore would not dissolve in aqueous solution even when first dissolved in DMF with subsequent addition to buffer. This made it incompatible both with the previously described fiber optic sensor method for testing antigenicity and with ELISA methods. It was, however, well suited to being tested using a sensor cartridge analogous to the type currently under development by IA, Inc. for measuring Pb^+. This fiber optic sensor cartridge was created using the membrane solution containing the new immunophore. An optical fiber was treated as previously described up to the point of silanization. The fiber was then drawn through a capillary tube containing 3μl of a membrane solution of previously described composition except that the ionophore was replaced by an equal molar amount of immunophore #2. When the sensor was exposed to pH 7 buffer without K^+ followed by the same buffer containing 10mM K^+ the sensor reading dropped by 645 pAmps. When the sensor was exposed to pH 7 buffer without K^+ followed by the same buffer containing 10mM K^+ and 2.46×10^{-7}M anti-estrone-3-glucuronide antibody, the sensor reading dropped by 574 pAmps. The difference of 71 pAmps in the two responses (a change of 11%) is consistent with what would be anticipated if the antibody bound to the immunophore and reduced its capacity to transport K^+ into the membrane.

IIc. Testing Strip Response In Presence And Absence Of Antibody.

Because the membrane solution made using Immunophore #2 loses most of its antibody binding capacity at the pH required to transduce a visual strip response to K^+, the membrane solution composition must be changed to return the K^+ response to pH 7 so that the antibody does not lose activity.

The membrane solution which was created as previously described using immunophore #2 in place of the ionophore. Strips were tested in 0.3M KCl at pH 5.28. Application of varying amounts of anti-E1-g ranging from 2.46×10^{-7}M to 1.25×10^{-5}M failed to alter strip response to 0.3M KCl. This raised immediate concerns as to whether the antibody was still viable at this lower pH. To test this, membrane was applied to a new optical fiber and the resulting sensor cartridge was exposed to pH 5.25 buffer followed by the same buffer containing 0.3M KCl. The sensor reading dropped by 919 pAmp. When the sensor was exposed to pH 5.25 buffer followed by the same buffer containing 0.3mM K^+ and 2.46×10^{-7}M anti-estrone-3-glucuronide antibody, the sensor reading dropped by 885 pAmps. The difference of 34 pAmps (a change of 3.7%) indicated less of a response to addition of antibody than was observed at pH 7 and 10mM KCl reported above.

The implication of the above observation was that the conditions required to see a visible color change with the new immunophore membrane reduced the change in response created by the presence of antibody.

Task III: Construction And Testing Of E1-g Measuring Strips.

> **Fiber optic sensors were used to measure the constants needed for simulating membrane performance on a computer. These computer simulations were used to successfully estimate membrane component formulations which provided test strips which were responsive to E1-g at physiological concentrations.**

As mentioned previously, the scope of this task was reduced to construction of only E1-g test strips. Design of an acceptable membrane composition was approached as originally described in the Phase I proposal so that data from fiber sensors bearing trial membranes could be assessed in comparison to theoretical models generated by software based upon the equations given in the Introduction (Ionopore Membrane Technology). This entailed a small change in experimental methodology. The software, created by our consultant Eric Bakker, is based upon plotting the parameter $1-\alpha$, which represents the fraction of potential chromionophores which are actually protonated, versus K^+ concentration given various assumptions for the constants K_{ex} and the composition of the membrane and pH of the experiment. The $1-\alpha$ calculation requires measurement of the completely deprotonated state of the membrane. Therefore each fiber experiment included measurement of fiber response at pH 9.5. Only the simulations which are directly relevant to the final membrane composition which was used to create viable E1-g test strips, responsive at levels of physiological interest are reported here.

Experimental Methods: A fiber was coated with the membrane which was used in the most recently described strip tests where pH 5.28 was required in order to observe a blue strip turn pink on exposure to KCl. The fiber was alternately incubated with .1m bis-tris propane buffer, pH 7 and that buffer containing varying concentrations of KCl. Reading on the pH 7 buffer as the solution was changed was taken when it remained constant within 5 pAmps over 3 minutes in order to insure that the previous K^+ sample had been fully washed out of the membrane. For K^+ and antibody solutions 100µl of the solution was injected and left in the sensor. Readings were taken every minute for 7 minutes. For each solution, α was determined by subtracting the reading with KCl from the previous reading in pH 7 buffer and dividing that quantity by the difference between the reading for pH 7 buffer and for pH 9.5 NH4OH. In experiments where antibody inhibition of immunophore response was being studied, the protocol was similar except that the varying concentrations of KCl were replaced with 0.4 M KCl, pH7 with and without varying amounts of antibody or antibody plus competing E1-g.

IIIa: Use Ionophore Membrane Simulation to Design E1-g Sensing Membrane.

Curve Fitting Procedure: The mathematical basis for the curve fitting program was derived by our consultant Dr. Eric Bakker. It follows:

Assume analyte A is neutral. Under this assumption, the ionophore changes binding constant to K^+ when binding to A and

$$\text{Beta(no A)} = [LK^+]/[L]\,[K^+]$$

$$\text{Beta (A)} = [LAK^+]/[LA]\,[K^+]$$

The distribution equilibrium of analyte A is given by: $D = A(org)/A(aq)$
Binding of A to L: $\text{BetaAL} = [AL]/[A]\,[L]$

Sample contains constant concentration of K^+ and H^+.

Starting point: no A

Ion-exchange equilibrium:

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20

$$K+(aq) + L(org) + CH+(org) = LK+(org) + C(org) + H+(aq)$$
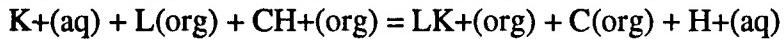
$$K_{exch} = \frac{[LK^+][C] \cdot a_{H^+}}{[L][CH^+] \cdot a_{K^+}} = K' \cdot \beta_{LK}$$

Limit: L saturated by A

$$K+(aq) + LA(org) + CH+(org) = LAK+(org) + C(org) + H+(aq)$$
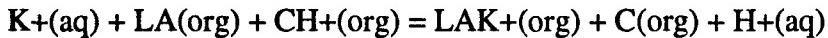
$$K'_{exch} = \frac{[LAK^+][C] \cdot a_{H^+}}{[LA][CH^+] \cdot a_{K^+}} = K' \cdot \beta_{LAK}$$

Intermediate Response Function:

Mass Balance:

$$L_T = [L] + [LA] + [LAK^+] + [LK^+]$$

Charge Balance:

$$R_T = [CH+] + [LAK^+] + [LK^+]$$

Distribution coefficient for A:

$$D_A = [A]/a_A$$

Binding constant betweeen A and L:

$$\beta_{AL} = [AL]/[A][L] = [AL]/(D_A \, a_A \, [L])$$

Therefore:

$$[AL] = \beta_{AL} \, D_A \, a_A \, [L]$$

Binding constant between A and LK+:

$$\beta_{ALK} = [ALK^+]/[A][LK^+] = [ALK^+]/(D_A \, a_A \, [LK^+])$$

Therefore:

$$[ALK^+] = \beta_{ALK} \, D_A \, a_A \, [LK^+]$$

Inserting into mass balance:

$$L_T = [L] + [LA] + [LAK^+] + [LK^+] = [L]\{1 + \beta_{AL} \, D_A \, a_A\} + [LK^+]\{1 + \beta_{ALK} \, D_A \, a_A\}$$

Inserting into charge balance:

$$R_T = [CH^+] + [LAK^+] + [LK^+] = [CH^+] + [LK^+]\{1 + \beta_{ALK} \, D_A \, a_A\}$$

Ion-exchange constant:

$$K_{exch} = \frac{[LK^+][C] \cdot a_{H^+}}{[L][CH^+] \cdot a_{K^+}} = K' \cdot \beta_{LK}$$

$$[CH^+] = (1-\alpha)\,C_T$$

$$[C] = \alpha\,C_T$$

$$[LK^+] = \{R_T - (1-\alpha)\,C_T\}/\{1 + \beta_{ALK}\,D_A\,a_A\}$$

$$[L] = (L_T - [LK^+])\,\{1 + \beta_{ALK}\,D_A\,a_A\})/\{1 + \beta_{AL}\,D_A\,a_A\}$$

$$[L] = (L_T - \{R_T - (1-\alpha)\,C_T\})/\{1 + \beta_{AL}\,D_A\,a_A\}$$

Grand total:

$$K_{exch} = \frac{a_{H^+}}{a_{K^+}} \cdot \frac{\alpha}{1-\alpha} \cdot \frac{R_T - (1-\alpha)C_T}{L_T - \{R_T - (1-\alpha)C_T\}} \cdot \frac{1 + \beta_{AL}D_A a_A}{1 + \beta_{ALK}D_A a_A}$$

Task: solve for a_A.

Simplify Problem:

$$K\,(1 + B\,a_A) = A\,(1 + C\,a_A)$$

$$K + K\,B\,a_A = A + A\,C\,a_A$$

$$K\,B\,a_A - A\,C\,a_A = A - K$$

$$a_A\,(K\,B - A\,C) = A - K$$

$$a_A = (A-K)/(K\,B - A\,C)$$

Since strips were responsive to K^+ at pH5 5.28, but not at pH 7, it is desirable to replace ETH 5294 with a chromionophore which would when the pH is 7, mimic performance of ETH 5294 at pH 5.28. It can be seen from Figure 7 that ETH 5350 comes close to fulfilling this requirement. The chromionophore of choice for future membranes thus became ETH 5350. This will permit use of pH 7 which will be a more desirable pH for the antibody.
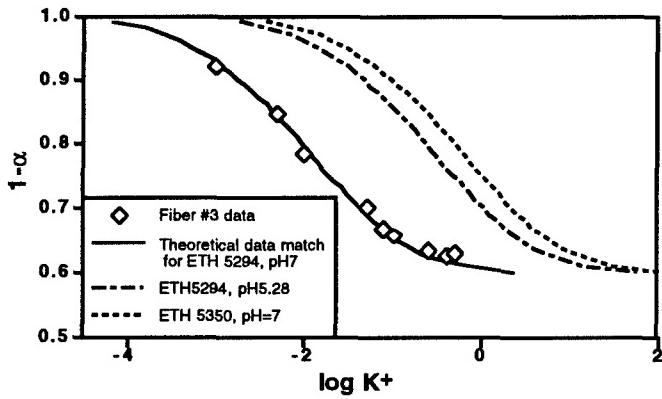

Figure 7: The response of a ETH 5294 Chromionophore immunophore coated fiber at pH=7 agrees with the theoretical predictions if $\text{Log}(K_{ex}) = -5.4$. Substitution of ETH 5350 at pH 7 should approximate performance with ETH 5294 at pH 5.28.

The second thing which is suggested by this simulation is that the amount of immunophore present is insufficient to produce a full range of variation in $1-\alpha$. While a fit was obtained using values for C and R corresponding to the measured amounts of chromionophore ETH 5294 and sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate actually present in the membrane, the amount of immunophore which had to be entered into the program (L=0.0072) in order to fit the theoretical curve to the data was less than what was measured out (0.11 mole/kg). The observation that the data flattens at a $1-\alpha$ of 0.6 can only be explained by an insufficiency of immunophore in relation the amount of chromionophore. Yet the weighed amount should have produced a full $1-\alpha$ range. It is possible that an unknown impurity prevents the weighed

amount of immunophore from representing the actual amount of active immunophore present in the membrane. This will have to be investigated during Phase II.

Simulation #2: Improving the Response Range of the Immunophore Membrane

A simulation of the amounts of membrane components needed to produce a membrane having an improved 1-α range at pH 7 using chromionophore ETH 5350, provided the formula for the Immunophore membrane which was used on successful test strips in Task IV.

Data shown in Figure #6 illustrates the K+ response of a fiber sensor bearing a membrane having composition based on the simulation shown. This membrane provided functional test strips in later tasks. The open circles represent the actual experimental data obtained using a fiber sensor having membrane composition: 0.0038 moles of ETH 5350, 0.0038 moles of sodium tetrakis[3,5-bis(trifluoromethyl)-phenyl] borate and 0.0176 moles of active immunophore #2 per kg of PVC-DOS. Again the active immunophore number used as L was less than the weighed amount (0.271 moles/kg). The experimental procedure was similar to that reported for Figure 7. The theoretical fit to experimental data resulted from assuming that log K_{ex} =-7. It can be seen that increasing the amount of immunophore II in the membrane and shifting to ETH 5350 has produced a less abbreviated range for 1-α.
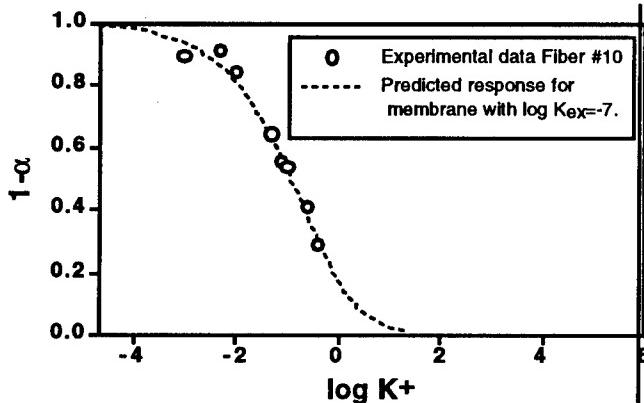
Figure 8: The response of the test strip membrane at pH = 7 agrees with theoretical membrane response predictions for a membrane having log(K_{ex}) =-7, L=0.0176, R=0.0038, and C=0.0038.

Fiber Optic Sensor Measurement Of Antibody Saturation Of The Membrane

The immunophore membrane, when applied to a fiber optic sensor showed saturation of the immunophore at an antibody concentration =1.2 x 10^{-7}M.

The same membrane solution was coated onto another fiber and the impact of varying concentrations of antibody on the response of the fiber noted. It can be seen from Figure 6 that the immunophore in the membrane appears to become saturated around an antibody concentration of 1.2 x 10^{-7} M anti-E1-g.

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23
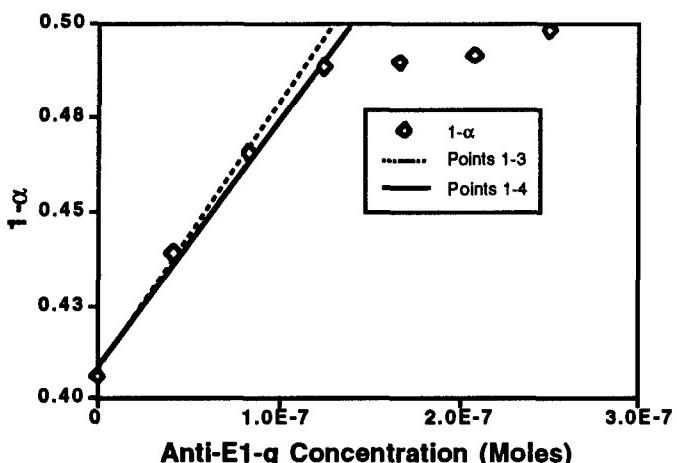
**Figure 9:** Saturation of available immunophore sites by anti-E1-g occurred at an anti-E1-g concentration of 1.2×10^{-7} Moles (near the value of point 4).

Use A Fiber Optic Sensor And Curve Fitting To Assess E1-G Response

Computer fit to sensor data determined that when antibody = 2.5×10^{-7} M, an ovulatory level of E1-g is discriminated from a non-ovulatory level.

In order to establish that the response shown in Figure 9 was due to binding between anti-E1-g and the immunophore in the membrane, and to assess the amount of antibody needed to obtain a differentiated response to physiological levels of E1-g another fiber optic sensor experiment was performed. A sensor was coated with the same membrane solution and incubated with a solution containing 2.5×10^{-7}M antibody along with either 0, 1.7×10^{-6}M or 4.3×10^{-7}M E1-g. Figure 10 shows curves that were fitted to the data points assuming K_{ex} and concentrations were identical to those used to generate Figure 8. $ß_{LK}$ and $ß_{LKA}$ were varied until a fit was obtained to match both of the points corresponding to 0 antibody and saturated antibody (1.2×10^{-7} M as determined in Figure 9) using the same $ß_{LK}$ and $ß_{LKA}$. These values were then held constant while A was varied until a curve was generated which matched a data point. That value of A was taken as the concentration of unbound antibody in the solution after competition with E1-g in the solution had proceeded for 10 minutes (experimental conditions and a reasonable amount of time to expect a user to tolerate between mixing a urine sample and getting a result).
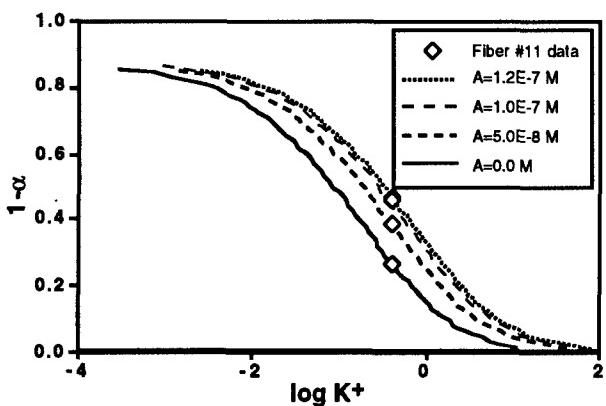
Figure 10: By matching theoretical membrane model response to different amounts of anti-E1-g (A) (using C=0.0038, R=0.0033, L=0.0176, Log(K_{ex})=-7, $B_1=2.0 \times 10^7$, $B_2=5.0 \times 10^4$) with the measured response of the immunophore membrane on Fiber#11 the amount of free antibody could be calculated.

Figure 11 shows a plot of the antibody concentrations computed above versus the 1-α response. That this presents a sigmoid response, typically seen in immunoassays (although the direction of this one is reversed due to the double competition nature of this method), indicates that the immunophore membrane presents a viable means of performing immunoassay. That the concentrations plotted were generated by competition of concentrations of E1-g within the range of physiological interest indicates that this membrane in a reasonable candidate for

producing test strips. This completes task IIIa and IIIb as redefined to include only an E1-g immunophore membrane. The data obtained in these experiment was used in calculating the number of moles of antibody which were applied to test strips in Task IV.


$$F(x) = d + a * \frac{\{\exp (b*(x+c)) - 1\}}{\{\exp (b*(x+c)) + 1\}}$$

a = -84.94
b = - 7.57
c = - 1.09
d = 84.61

R^2 = 0.9905
SSE = 0.0098

y-axis: 1-α
x-axis: Antibody Concentration (x10^7)
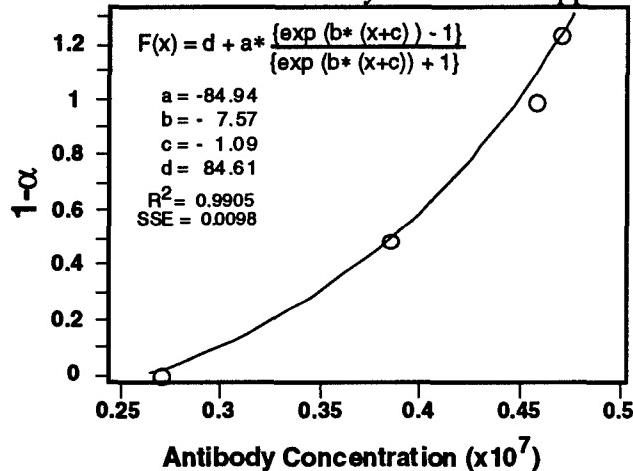
Figure 11: There is a sigmoid relationship between (1-α) and the concentration of antibody generated by competition from E1-g.

IIIb. Synthesize Required Immunophores.

Synthesis of an immunophore for E1-g has been described in Task IIa. After incorporating the experimental data obtained in Task IIc into the membrane model, E1-g immunophore membrane solutions were formulated having appropriate ionophore molecule concentrations. These membrane solutions were used in Task IIIc for fabricating E1-g test strips.

IIIc: Create Test Strips According To Design Specifications.

> **Test strips were made using membrane of composition developed in Task IIb. Antibody was applied to strips in amounts based upon the antibody concentration which was shown to distinguish relevant E1-g concentrations**

The test strip consists of a strip of Whatman chromatography paper, grade 20 which was 4mm x 10 cm. The antibody was anti-estrone-3-glucuronide monoclonal antibody clone #278-17144 obtained through OEM Concepts Inc., Toms River, New Jersey. The composition of the first batch of membrane solution was:
Membrane solution batch #1:
* 200 µl of a solution plastic consisting of 20 mg of polyvinyl chloride (high molecular weight) & 40 mg of bis(2-ethylhexyl) sebacate and 1 ml. of redistilled tetrahydrofuran

* 22 µl of a solution containing 1.167 mg Chromionophore III ETH 5350 in 1 ml. redistilled tetrahydrofuran

* 5 µl of a solution containing 1.67 mg. of sodium tetrakis[3,5-bis(trifluoromethyl) phenyl]borate in 0.2 ml. of tetrahydrofuran

* 150 µl of a tetrahydrofuran solution containing 3 mg of the immunophore described above.

All reagents listed above were obtained from *Fluka Chemica*.

Membrane solution batch #2: In order to increase the color contrast on the strips a second batch of membrane was made up as follows:
  * 150 µl of a solution plastic consisting of 20 mg of polyvinyl chloride (high molecular weight) & 40 mg of bis(2-ethylhexyl) sebacate and 1 ml. of redistilled tetrahydrofuran

* 30 µl of a solution containing 1.167 mg Chromionophore III ETH 5350 in 1 ml. redistilled tetrahydrofuran

- 10 μl of a solution containing 1.67 mg. of sodium tetrakis[3,5-bis(trifluoromethyl) phenyl]borate in 0.2 ml. of tetrahydrofuran

- 100 μl of a tetrahydrofuran solution containing 3 mg of the immunophore described above.

Making the test strips

3 μl of membrane solution was spread on the surface of the strip in an area occurring between 2.5-4.0 cm from the bottom of the strip, or it was applied in a series of 3 dots in the same area each time dispensing 1 μl of membrane solution. Solutions containing between 2.5×10^{-10} to 3×10^{-9} moles of antibody in 1.0 μl of 0.1M bis-tris propane buffer, pH 7.002 were used. Use of these antibody concentrations was arrived at by multiplying 2.5×10^{-7} M by the ratio of volumes of solutions used in the sensor experiments as compared to the strips and varying the antibody by a factor of 10 around that amount as calculated in moles applied to the strip. Volumes of antibody spotted ranged from 0.5-1.9 μl of antibody solution. dotted onto the strip at a point 2 cm from the bottom of the strip. When all solutions had dried on the strip, the strip was mounted vertically so that its bottom touched the bottom of a well of an ELISA plate, the strip being held perpendicular to the well. The strip did not contact any other surface below the mounting point. 40 μl of 0.1M bis-tris propane buffer, pH 7.002 was placed in the well and allowed to rise up the strip chromatographically, distributing the antibody over the surface of the membrane where it was bound by the immunophore. This completed Task IIIc as limited to E1-g.

Task IV: Test the strips in buffer and urine containing relevant concentrations of hormones.

Buffer samples were created by adding 10 μl of either 2 ng/μl, 8 ng/μl or 100 ng/of E1-g, in aqueous solution to 990 μl. of 0.1M KCl in 0.1M bis-tris propane buffer, pH=7.002 to give sample concentrations of E1-g of 20 ng/ml (4.3×10^{-7}M) or 80 ng/ml (1.7×10^{-6}M). 20 μl of this was chromatographed up the strip and the height at which the strip changed from blue to pink correlated with the concentration of estrone-3-glucuronide in the sample. Example strips demonstrating 20ng/ml and 80ng/ml samples of estrone-3-glucuronide are shown in Figures 12 and 13.

20   80

(a)

20   80

(b)
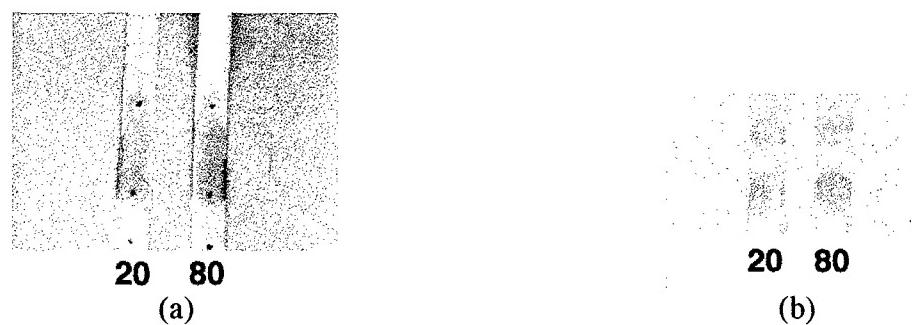
Figure 12: (a) Test Strip 9-10-96 made from Batch #1 membrane solution (used for fiber tests shown above) showed noticeable differences in the location of the color changes when tested in buffer using 20 ng/ml (non-ovulatory) and 80 ng/ml (ovulatory) levels of E1-g. (b) Test Strip 9-11-96 #1 made from Batch #2 of membrane also shows this difference.

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26

0　20　80　　　0　20　80　1000　　　20　80　20　80　1000
(a)　　　　　　　(b)　　　　　　　(c)
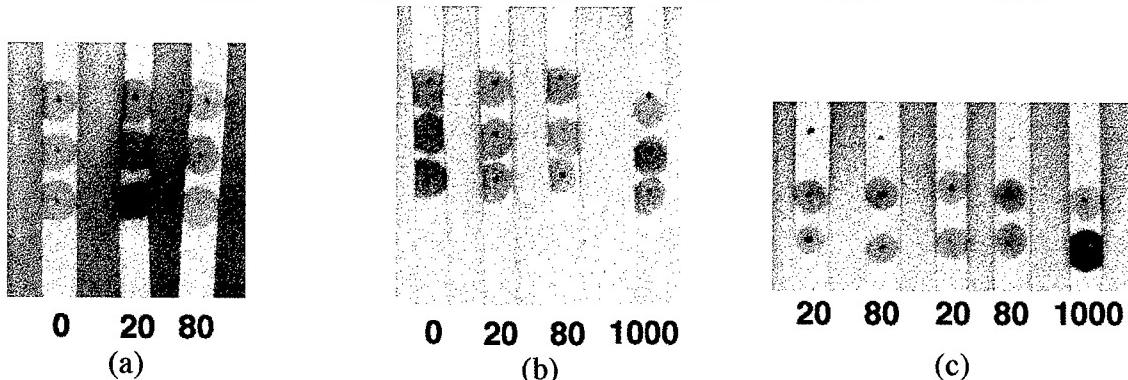
Figure 13: Test Strips made using Batch #2 membrane solution showed more distinctive color changes when tested in buffer solutions containing 0, 20, 80 and 1000 ng/ml of E1-g. Test Strip results for various amounts of assay anti-E1-g are shown in (a) for Test Strip 9-13-96 #3. (anti-E1-g = 1.0×10^{-9} Moles), (b) for Test Strip 9-13-96 #3.(anti-E1-g = 2.0×10^{-9} Moles), and (c) Test Strip 9-14-96 #1 (anti-E1-g = 3×10^{-9} Moles).

Strips using 2.5×10^{-10} and 5×10^{-10} moles of antibody in the spot did not produce useful distinctions and are not shown. The darker spot on the bottom of the strip labeled 20 in (a) did not look that dark visually . Apparent differences in color tone between (a), (b) and (c) are artifacts of the process of scanning strips into the computer.


B　N　O　　　　B　O　N　　　　B　N　N　O
(a)　　　　　　　(b)　　　　　　　(c)
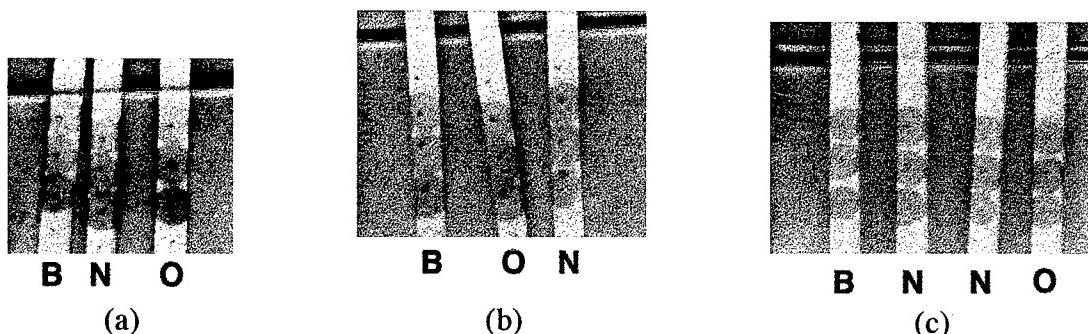
Figure 14: The Phase I Immunophore Test Strips discriminate between non-ovulatory and ovulatory urine samples. Results for Immunophore Test Strips fabricated using Batch 4 membrane solution are shown in (a) for Strip Test 9-23-96 #1 in Buffer (B), non-ovulatory urine (N - 8.5 ng/ml), and ovulatory urine (O - 85.9 nm/ml), (b) for Test Strip 9-23-96 #2 in Buffer (B), ovulatory urine (O - 85.9 nm/ml), and non-ovulatory urine (N - 8.5 ng/ml), and (c) for Test Strip 9-24-96 #1 in Buffer (B), non-ovulatory urine (N- 5.5 ng/ml), and ovulatory urine (O - 39.5 ng/ml).

Testing Urine Samples: Urine samples which had been previously assayed for E1-g by means of ELISA and stored frozen were thawed and mixed. Ovulatory and non-ovulatory samples were chosen from two different subjects. A KCl-pH7 buffer solution was made up to be 10 times the concentration of the KCl-buffer used above. 1 part of this solution was added to 9 parts of samples to be tested. Results are shown in Figure 14.

Strips made using distinct membrane spots were observed to be more likely to give the correct discrimination between samples than strips made using a continuous membrane layer. This effect is attributed to the extreme volatility of tetrahydrofuran which made it more difficult to apply a uniformly thick continuous membrane layer across different strips than to apply the membrane in a series of distinct 1 μl spots along each strip. In addition, factors which affect the rate at which liquid wicks up a filter paper strip were seen to affect results and sometimes cause strips to give inaccurate comparisons between samples. These factors included including:

1) Variations in the way the liquid sample touched the strips at the bottom of the ELISA

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27

wells,

2) Variations in the distribution of the spread of the antibody solution applied to the strip,
3) Variations in the angle of contact between the strip and the liquid,
4) Variations in position of the strip in relation to a window with sun shining in. The strip on the outer edge near the window was most likely to give incorrect results.
5) Variations in the rate at which the liquid chromatographed up the strips.
6) Variations in the shape and density of the membrane which forms on the paper.

While work still needs to be done to improve the uniformity of membrane thickness, the reproducibility of liquid wicking through the strip and to extend the immunophore technology to measure other hormones, our Phase I results are extremely encouraging. Although we were unable to adequately control the liquid wicking rate through the porous paper backing of the test strips, during the Phase I effort, the El-g test strips yielded the correct results in urine roughly 75% of the time. Moreover, we have shown that by adjusting the composition of the ionophore membrane constituents, visible color changes may be made to occur at a specific urine hormone concentration levels. This will allow test strip assays to be developed which provide a quantitative measure of urine hormone levels.

These tests results represent a successful completion of all of the proposed Phase I tasks as applied to El-g and provide a demonstration of the feasibility of the technology. Since Pd–g is simpler to measure than El-g, we are certain that ionophore membrane strip tests can also be fabricated for both El-g and Pd-g..

Task V: Compile data, assess necessary steps to achieve a reproducible product and write the Final Report.

This task has been completed with the completion of this report and submission of the Phase II proposal.

Conclusions

The feasibility of creating test strips based upon mediation of immunological binding by a specifically created "immunophore" molecule has been demonstrated. An immunophore has been synthesized which transports potassium ion into a PVC membrane and competes with a chromionophore for a lipophilic counter-ion to create an ionophore membrane which gives a quantifiable color response to changes in potassium ion in solution. The immunophore alters its potassium transport capacity upon binding of its antigenic portion (in this case, E1-g) to an antibody. Response characteristics of membranes incorporating this immunophore have been measured using a fiber optic sensor and its capacity to transduce the amount of E1-g in a solution into a measurable change in color of the chromionophore has been validated. The membranes behave in accordance with theoretical expectations with one exception. The amount of immunophore which must be entered into the theoretical program in order to match the actual data is consistently 6.5% of the actual weighed amount of immunophore. Although this doesn't cause any difficulties with the functioning of the membrane it does suggest that additional investigation into the exact composition of the immunophore preparation is needed in Phase II.

Test strips to which the immunophore membrane and anti-E1-g had been applied responded to buffer and urine samples with the correct discrimination between ovulatory and pre-ovulatory levels of E1-g in about 75% of the tests which were run. When tests were not accurate, it was usually possible to identify something in the manner of the chromatographic distribution of the sample over the membrane which looked different from the ones which gave accurate responses. For example, the liquid traveled more slowly or the membrane looked thicker or didn't quite distribute across the entire strip. These are the types of things which should be amenable to correction by development of methods for control of these factors which will be developed in Phase II work.

The potential of this technology is exciting. It can make possible semiquantitative measurements, outside of a laboratory, of a large number of hormones, toxins, contaminants, drug and metabolites which currently require laboratory immunoassay to provide useful information. It is particularly well suited to field applications in that no instrumentation is required in order to detect and semiquantify analytes having concentrations at least down to $1 \times 10^{-7}M$. Lower limits have not yet been explored because they are not required by the current E1-g application. Establishing the potentially useful range of the technology will be accomplished in Phase II work.

The specific application, identification of a woman's fertile period, has tremendous potential for becoming a profitable product as an over-the-counter aid in family planning. Because it requires no instrumentation it presents an ideal product for less industrial counties. IA, Inc. is very enthusiastic about the potential of this product and has applied for patents to cover both the general technology as applied to test strips and the specific application to fertile period identification. An addition patent covering application of immunophore membrane technology to fiber optic sensors is in the process of being written. The latter development which occurred as a side product in the course of developing the strip test is in itself a product of potential for applications requiring more both rapid results and precise quantification.

This project has permitted IA, Inc. to develop a product with exciting potential. We thank the Army for providing the support which made this possible and we hope the work will be deemed worthy of Phase II support.

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29

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